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TITLE: Restoration of Wild-Type Activity to Mutant p53 in Prostate Cancer: A Novel Therapeutic Approach

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14. ABSTRACT A summary is presented of research performed during the second year of a project to explore approaches to restore wild-type function on mutant p53 proteins found in human prostate tumors. p53 mutant proteins derived from prostate tumors are being characterized to determine their suitability. Three specific aims are being pursued. The first is characterizing the interaction of p53 with two distinct classes of its response elements. The second aim is determining the role of mutant p53 proteins in prostate cancer cell proliferation. The final aim is to explore approaches to restore wild-type function to mutant p53 proteins found in prostate cancer. This is a chemical biological approach with the goal of restoring wild-type function to prostate tumor-derived mutant p53 proteins. This research is geared towards preclinical development of a highly targeted therapy for human prostate cancer.					
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Introduction

The notion that mutant p53 proteins can regain wild-type function is the focus of the ongoing research funded by this IDEA award from the Department of Defense Prostate Cancer Research Program. Previous studies from our laboratory demonstrated that genomic binding sites for p53 can be classified into two classes (Resnick-Silverman et al., 1998; Thornborrow and Manfredi, 1999; St. Clair et al., 2004). As tumor-derived p53 mutant proteins have typically lost the ability to bind to DNA in a sequence-specific manner, approaches to restore wild-type function will require a detailed understanding of the molecular basis for DNA binding by p53. Thus, the goals of this research are to characterize the two distinct classes of p53 binding sites, to perform proof-of-principle studies for restoration of wild-type function to mutant p53, and to explore approaches to restore wild-type function which may have therapeutic utility. This report covers the second year of a three-year funding period.

Body

Task 1. Validate the existence of different classes of p53 response elements (Months 1-12)

During the first year of funding,, seventeen different p53 response elements were used to generate luciferase reporters. These were used to confirm the existence of two distinct groups of p53 binding sites using transfection studies in p53-null cells as well as the use of a cell line with tetracycline regulated expression of p53. These studies demonstrated that DNA binding did not correlate with transcriptional activation for a subset of p53 response elements and form the basis for a manuscript that has been submitted for publication (Giono et al., see below). Chromatin immunoprecipitation (CHIP) studies have confirmed that p53 can occupy a subset of sites with similar affinity in cells after treatment with DNA damaging agents (Figure 1). This validates the findings reported last year using in vitro DNA binding assays. Thus, p53 can interact with a subset of its binding sites to a similar extent and yet its ability to induce transcriptional activation varies. Consistent with the notion that p53 adopts distinct conformations on different binding sites, further experiments have shown that the C-terminus of p53 can regulate binding to the set of sites which are robustly activated, but is dispensable for binding to sites that are weakly activated. Taken together, these studies have indeed validated the existence of different classes of p53 response elements.

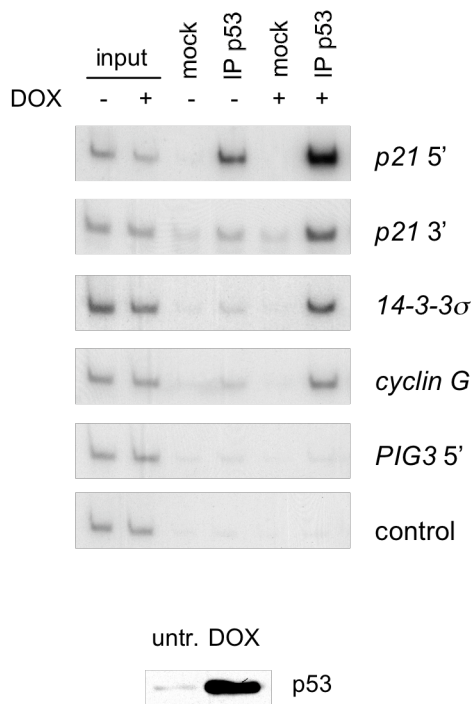


Figure 1. p53 occupies its response elements to similar extent after DNA damage. (A) Cells were treated with 0.5 µg/ml doxorubicin for 12 h. Chromatin immunoprecipitation assays were performed to detect the association of p53 to the response elements in several target genes and a control unrelated region. (B) p53 and actin protein levels in the extracts were assayed by immunoblotting.

Task 2. Determine whether p53 adopts distinct conformations on subsets of its response elements (Months 6-15)

It was previously reported that conditions for controlled proteolysis of p53 had been established. Effects of different DNA binding sites on proteolysis of p53 were then examined (Figure 2). Each of the sites in

the p21 promoter is representative of a distinct subset of p53 response elements. The effect of the mAb 421 on the in vitro DNA binding of p53 to several of its known response elements was tested. These included sites in the promoters of the cyclin G, bax, and cdc25C genes as well as a novel site identified in the first intron of the human p21 gene. This latter site can be shown to bind specifically to p53 in vitro and also confer p53-dependent transcriptional activation on a luciferase reporter containing a minimal adenoviral E1b promoter. The p21 5', p21 intron, and cyclin G binding sites all showed enhanced binding to p53 in the presence of mAb 421, whereas the p21 3', cdc25C, and bax sites did not (Figure 2A). As a control, the mAb 1801 did not alter the binding affinity of p53 for any of the sites. The binding affinities of various sites for the chymotrypsin fragment gave similar results as incubation with mAb 421. The partially digested p53 binding patterns of the cdc25C and bax sites were similar to that of the p21 3' site, showing decreased binding with increased digestion as compared with the enhanced binding to the p21 5' site at the 1-5 ng chymotrypsin level (Figure 2B). However, the p21 intron and cyclin G binding patterns were similar to the p21 5' site with enhanced binding to the partially digested p53. Thus, the p21 5' site is representative of p53 binding sites that show enhanced binding with mAb 421 or partial chymotrypsin digestion. In contrast, the p21 3' site is representative of p53 binding sites in which neither the mAb 421 nor the chymotrypsin fragment confers enhanced binding. These findings form the basis for a manuscript which is being revised for J. Mol. Biol. (Maurer et al.).

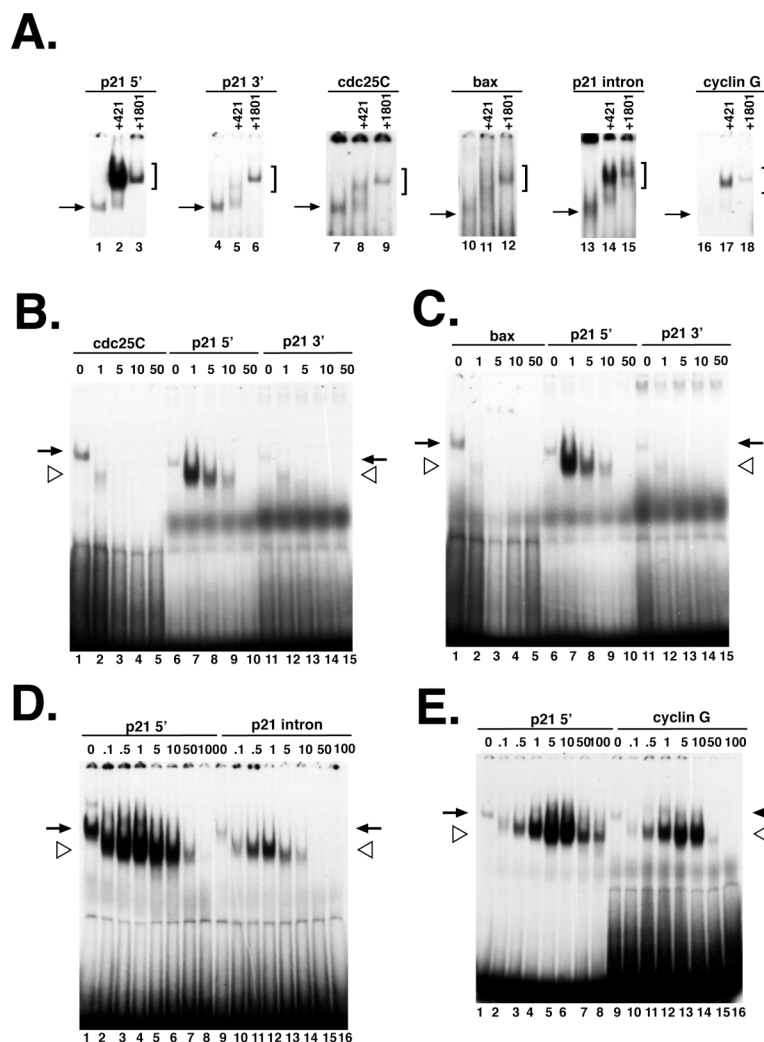


Figure 2. Each of the sites in the p21 promoter is representative of a distinct subset of p53 response elements. (A) EMSA were performed using oligonucleotides corresponding to either the p21 5' site (lanes 1-3), the p21 3' site (lanes 4-6), the cdc25C site (lanes 7-9), the bax site (lanes 10-12), the p21 intronic site (lanes 13-15), or the cyclin G promoter site (lanes 16-18) as radiolabeled probe. 10 ng of purified p53 was incubated with no additions (lanes 1, 4, 7, 10, 13, 16), or in the presence of 800 ng of mAb 1801 (lanes 2, 5, 8, 11, 14, 17), or mAb 421 (lanes 3, 6, 9, 12, 15, 18). The arrows to the left indicate the positions of the p53-DNA complex and the brackets to the right show the super-shifted complex containing antibody, p53, and DNA. (B-E) 10 ng of purified p53 was digested with increasing amounts of chymotrypsin (0-100 ng, as indicated) for 20 minutes and processed as described in Experimental Procedures. EMSA was performed with the indicated sites as radiolabeled probes. For each autoradiogram, the black arrows indicate the position of the DNA complex with intact p53 and the open arrowheads mark the position of the DNA complex containing chymotrypsin-digested p53.

Task 3. Determine the significance of distinct classes of p53 response elements (Months 16-27)

Conditions have been established to detect an interaction between p53 and either of its coactivators CBP or p300 in solution (Figure 3). Studies are now underway to examine the ability of p53 to interact with candidate co-factors such as these in pull-down assays using examples of each class of p53 response element and to explore the identities of novel proteins that interact with p53 in a binding site-dependent manner.

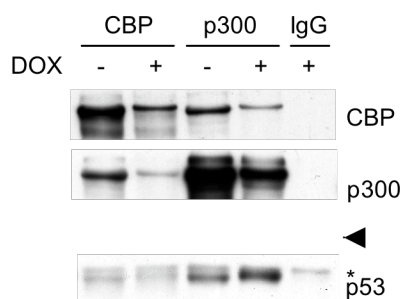


Figure 3. The interaction of p53 with the co-activators CBP and p300 is not affected by DNA damage. Cells were treated with 0.1µg/ml doxorubicin for 24 hr. Cell lysates were then subjected to immunoprecipitation with either anti-CBP, anti-p300, or a control IgG. Immunoblotting for CBP, p300, and p53 was then performed. The asterisk indicates a non-specific band that is detected in all lanes.

Task 4. Determine the role of p53 in mutant p53-expressing DU145 prostate cancer cells (Months 1-27)

The expression of mutant p53 in DU145 prostate cancer cells was ablated using transient transfection with siRNA oligonucleotides against p53 (Figure 4A). Substantial reduction in mutant p53 expression did not affect the proliferation of these cells as determined by flow cytometric analysis (Figure 4A). DU145 cells were then co-transfected with a plasmid expressing a short hairpin RNA against p53 as well as one that confers resistance to puromycin. Drug-resistant colonies were isolated three weeks later and analyzed for mutant p53 expression (Figure 5B). Clones that showed loss of p53 expression were readily detected. The levels of p53 in six such clones are shown in Figure 4B. No effect on cell proliferation was detected when these clones (D6-D10) were compared to control clones (D1-D5). A representative example is shown in Figure 3B for clone D6. These results suggest that mutant p53 expression is not required for DU145 prostate cell proliferation. We are now establishing approaches to restore wild-type p53 expression in DU145 cells depleted of endogenous p53 expression and to characterize these DU145 cells with restored wild-type p53 expression. These studies will provide proof-of-principle for whether methods to restore wild-type activity to cells expressing mutant p53 will be useful.

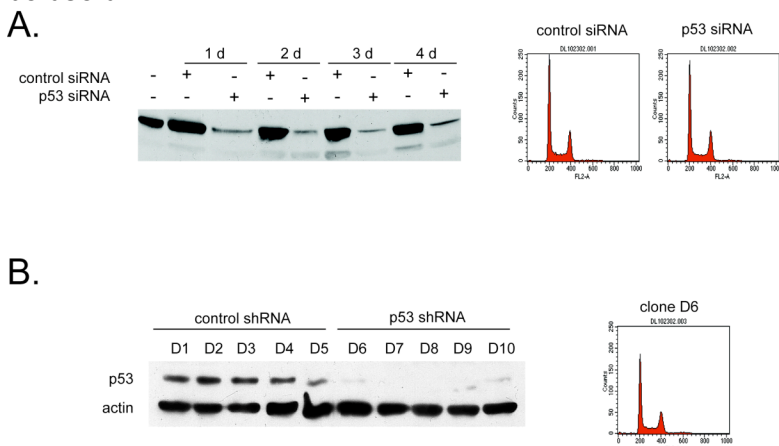


Figure 4. Ablation of p53 expression does not affect proliferation of DU145 prostate cancer cells. (A) DU145 cells were transiently transfected with siRNA oligonucleotides against p53. Immunoblotting for p53 was performed at 1-4 days after transfection (left panel). Cells at day 4 were stained with propidium iodide and subjected to flow cytometric analysis (right panel). (B) DU145 cells were transfected with a plasmid expressing an shRNA against p53 as well as one conferring puromycin resistance. Drug-resistant colonies were isolated and subjected to immunoblotting for p53 (left panel). One such clone (D6) was analyzed by flow cytometry (right panel).

Task 5. Examine the role of prostate tumor-derived mutant p53 proteins in regulating cell proliferation (Months 15-31)

PC-3 cells were transfected with expression plasmids expressing wild-type p53 or the mutants P223L or V274F. These latter two mutants are the p53 proteins which are expressed in the DU145 cell line. These plasmids were co-transfected with one that confers puromycin resistance and colony assays were performed. Wild-type p53 completely suppressed colony formation in this assay whereas the two mutants showed impaired ability to do so (Figure 5A). Levels of expression of the various p53 proteins were shown to be comparable by immunoblotting (Figure 5B). Interestingly, the P223L mutant had comparable activity as wild-type in activating transcription of a luciferase reporter containing the human p21 promoter. In contrast, V274F failed to do so (Figure 5C). PC3 cells expressing various prostate-specific mutant p53 are now being established and will be characterized as described in the research plan.

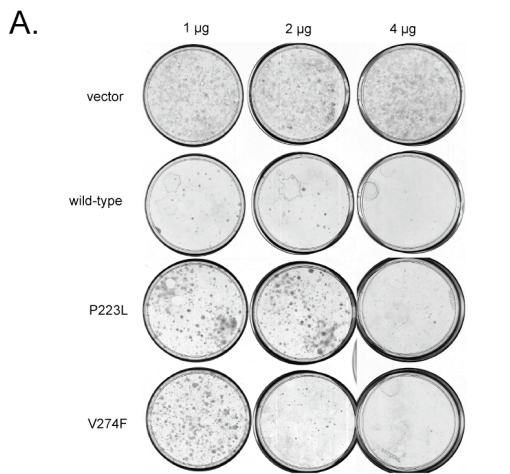
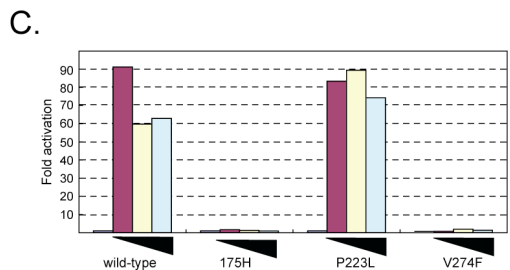
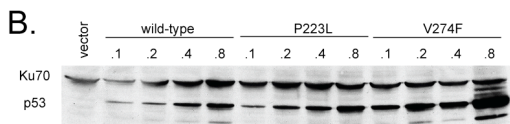


Figure 5. The mutant p53 proteins expressed by DU145 prostate cancer cells show impaired ability to inhibit cell proliferation. (A) p53-null PC3 cells were co-transfected with plasmids expressing wild-type p53 or the mutants P223L or V274F with a plasmid conferring puromycin resistance. After 3 weeks, colonies were stained with crystal violet. (B) Levels of expression of each of the p53 proteins were detected by immunoblotting after transient transfection with the appropriate expression plasmid. (C) A luciferase reporter containing the full-length human p21 promoter was co-transfected with expression plasmids expressing the indicated p53 proteins. After 24 hr, cells were lysed and luciferase activity was detected.



Task 6. Characterize previously identified methods to restore wild-type function on prostate tumor-derived mutant p53 proteins (Months 24-36)

This task is to be performed in the final funding year.

Task 7. Establish approaches to screen for compounds which restore the ability of prostate tumor-derived mutant p53 proteins to activate transcription (Months 1-36)

During the first year of funding, GFP reporters containing the p21 promoter with deletions of each p53 site were generated. Establishment of stable transfectants of PC3 cells (Task 5) is ongoing. As soon as this is complete, a cell-based assay for monitoring GFP read-out from p21 reporters will be validated. Finally, a library of compounds will be screened in these cell-based assays .

Key Research Accomplishments

- Validated the existence of two distinct classes of p53 response elements that are differentially regulating by the C-terminus of p53
- Showed that DNA binding affinity is not the major determinant for transcriptional activation of a subset of targets p53 implicating a role for conformation
- Established methods for limited proteolysis of p53 and used this approach to validate the notion that p53 adopts different conformations on subsets of its genomic binding sites
- Determined that expression of mutant p53 is not required for ongoing proliferation of DU145 cells
- Demonstrated that the mutant proteins expressed by DU145 cells, P223L and V274F, are hypomorphic alleles in that they have impaired activity but do retain some ability to inhibit cell proliferation

Reportable Outcomes

- Maurer, M., Resnick-Silverman, L., Thornborrow, E., and Manfredi, J.J.. The C-terminal region of p53 selectively regulates binding of p53 to a distinct subset of its genomic response elements. *J. Mol. Biol.*, in revision.
- Giono, L.E., Resnick-Silverman, L., Lufkin, D.J., and Manfredi, J.J. DNA binding affinity is not the major determinant for transcriptional activation of a subset of targets by the tumor suppressor p53: a role for conformation and the C-terminus. Submitted.
- Beck, D., Liu, W., Resnick-Silverman, L., and Manfredi, J.J. Two hypo orphic mutants of the p53 tumor suppressor synergize to abrogate the DNA damage response in prostate cancer cells. In preparation.
- Resnick-Silverman, L., Beck, D., Liu, W., and Manfredi, J.J. The tumor suppressor p53 prevents apoptosis in response to chemotherapeutic agents in prostate cancer cells. In preparation.
- Established clones of DU145 cells which lack expression of endogenous p53 protein
- Established clones of PC3 cells which express mutant p53 proteins derived from human prostate tumors

Conclusions

The main goal of these studies is to determine the feasibility of an approach to restore wild-type activity to mutant p53 proteins derived from human prostate tumors. Studies to date have confirmed the existence of different classes of p53 binding sites and provided feasibility for many of the planned approaches to be utilized in the final year of funding. This represents a preclinical development effort that will establish whether such a therapeutic approach is worth pursuing. As part of these ongoing studies, important insights into mutant p53 biology as it relates to human prostate cancer are also being gained.

References

Resnick-Silverman, L., S. St Clair, M. Maurer, K. Zhao, and J. J. Manfredi. 1998. Identification of a novel class of genomic DNA-binding sites suggests a mechanism for selectivity in target gene activation by the tumor suppressor protein p53. *Genes Dev* 12:2102-7.

Thornborrow, E. C., and J. J. Manfredi. 1999. One mechanism for cell type-specific regulation of the bax promoter by the tumor suppressor p53 is dictated by the p53 response element. *J Biol Chem* 274:33747-56.

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Appendices

None.